Activation of WD Repeat and High-Mobility Group Box DNA Binding Protein 1 in Pulmonary and Esophageal Carcinogenesis

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Abstract

Purpose: We attempted to identify novel biomarkers and therapeutic targets for lung and esophageal cancers.

Experimental Design: We screened for genes that were overexpressed in a large proportion of lung and esophageal carcinomas using a cDNA microarray representing 27,648 genes or expressed sequence tags. A gene encoding WDHD1, a WD repeat and high-mobility group DNA binding protein 1, was selected as a candidate. Tumor tissue microarray containing 267 archival non-small cell lung cancers and 283 esophageal squamous cell carcinomas (ESCC) was used to investigate the clinicopathologic significance of WDHD1 expression. The role of WDHD1 in cancer cell growth and/or survival was examined by small interfering RNA experiments and cell growth assays. The mechanism of WDHD1 activation through its phosphorylation in cancer cells was examined by immunoprecipitation and kinase assays.

Results: Positive WDHD1 immunostaining was associated with a poor prognosis for patients with non–small cell lung cancer (P = 0.0403) as well as ESCC (P = 0.0426). Multivariate analysis indicated it to be an independent prognostic factor for ESCC (P = 0.0104). Suppression of WDHD1 expression with small interfering RNAs effectively suppressed lung and esophageal cancer cell growth. In addition, induction of the exogenous expression of WDHD1 promoted the growth of mammalian cells. AKT1 kinase seemed to phosphorylate and stabilize the WDHD1 protein in cancer cells.

Conclusions: WDHD1 expression is likely to play an important role in lung and esophageal carcinogenesis as a cell cycle regulator and a downstream molecule in the phosphoinositide 3-kinase/AKT pathway, and that WDHD1 is a candidate biomarker and a promising therapeutic target for cancer. Clin Cancer Res; 16(1); 226–39. ©2010 AACR.
Translational Relevance

Because there is a correlation between WDHD1 expression and poor prognosis for patients with lung and esophageal carcinomas, and multivariate analysis indicated it to be an independent prognostic factor for esophageal carcinomas, WDHD1 positivity in resected specimens could be an index that provides information useful to physicians in applying adjuvant therapy and intensive follow-up to the cancer patients who are likely to suffer a relapse. Because WDHD1 should properly be classified as a typical cancer testis antigen and is likely to play an important role as a key component of the phosphoinositide 3-kinase/AKT pathway in cancer proliferation, the selective inhibition of WDHD1 expression and/or the targeting of enzymatic interaction between AKT1 and WDHD1 by small molecule compounds could be a promising therapeutic strategy that is expected to have a powerful biological effect on cancer with a minimal risk of adverse events.

WDHD1 in Pulmonary and Esophageal Carcinogenesis

Materials and Methods

Cell lines and tissue samples. The human lung cancer cell lines used in this study were as follows: lung adenocarcinomas (ADC) NCI-H1781, NCI-H1373, LC319, AS549, and PC14; lung squamous cell carcinomas (SCC) SK-MES-1, NCI-H2170, NCI-H520, NCI-H1703, and LU61; a lung large-cell carcinoma LX1; and small-cell lung cancers (SCLC) SBC-3, SBC-5, DMS273, and DMS114 (Supplementary Table S1). The human esophageal carcinoma cell lines used in this study were as follows: 10 SCC cell lines (TE1, TE2, TE3, TE4, TE5, TE6, TE8, TE9, TE10, and TE11) and one ADC cell line (TE7; ref. 42). All cells were grown in monolayer in appropriate medium supplemented with 10% FCS and maintained at 37°C in humidified air with 5% CO2. Human small airway epithelial cells (SAEC) used as a normal control were grown in optimized medium (small airway growth medium) from Cambrex Bioscience, Inc. Primary non-SCLC (NSCLC) and ESCC tissue samples as well as their corresponding normal tissues adjacent to resection margins from patients having no anticancer treatment before tumor resection had been obtained earlier with informed consent (7, 11, 12, 15). All tumors were staged on the basis of the pathologic tumor-node-metastasis classification of the International Union Against Cancer (43). Formalin-fixed primary lung tumors and adjacent normal lung tissue samples used for immunostaining on tissue microarrays had been obtained from 267 patients undergoing curative surgery at Hokkaido University and its affiliated hospitals (Sapporo, Japan; please see Supplementary Table S2A). A total of 283 formalin-fixed primary SCCs and adjacent normal esophageal tissue samples near to resection margins had also been obtained from patients undergoing curative surgery at Hokkaido University Hospital and Keiyukai Sapporo Hospital (Sapporo, Japan; Supplementary Table S2B). To be eligible for this study, tumor samples were selected from patients who fulfilled all of the following criteria: (a) patients suffered primary NSCLC or ESCC with histologically confirmed stage (only pT1 to pT3, pN0 to pN2, and pM0); (b) patients underwent curative surgery, but did not receive any preoperative treatment; (c) among them, NSCLC patients with positive lymph node metastasis (pN1, pN2) were treated with platinum-based adjuvant chemotherapies after surgery, and ESCC patients with pN positive were treated with adjuvant chemotherapy using both platinum and 5-fluorouracil after surgery, whereas patients with pN0 did not receive adjuvant chemotherapies; and (d) patients whose clinical follow-up data were available. This study and the use of all clinical materials mentioned were approved by individual institutional ethics committees.

Semiquantitative reverse transcription-PCR. A total of 3 μg aliquot of mRNA from each sample was reversely transcribed to single-stranded cDNAs using random primer (Roche Diagnostics) and SuperScript II (Invitrogen). Semiquantitative reverse transcription-PCR (RT-PCR) experiments were carried out with the following sets of synthesized primers specific to WDHD1 or with β-actin

WDHD1 is a human homologue of Ctf4 in *Saccharomyces cerevisiae*, which was originally identified by screening for mutant genes affecting chromosome transmission fidelity (38). Later studies indicate that Ctf4 is required for sister chromatid cohesion and interacts with DNA polymerase (38). Later studies indicate that Ctf4 is required for sister mutant genes affecting chromosome transmission fidelity.
(ACTB)—specific primers as an internal control: WDHD1, 5′-AGTGAAGGAACTGAAAGCAAGAG-3′ and 5′-ATCTCATTACTTCCTTAGG-3′; ACTB, 5′-GAGGTGATACGCAATTGCTTCTG-3′ and 5′-CAAGTCACTGTCAGGG-TAACG-3′. PCRs were optimized for the number of cycles to ensure product intensity to be within the linear phase of amplification.

**Northern blot analysis.** Human multiple tissue blots covering 23 tissues (BD Bioscience) were hybridized with an [α-32P]-dCTP-labeled, 535-bp PCR product of WDHD1 that was prepared as a probe using primers 5′-CTCTGATTCCAAAAGCCGAAG-3′ and 5′-ATCCATTACTTCCTAGGTCAC-3′. Prehybridization, hybridization, and washing were done following the manufacturer’s specifications. The blots were autoradiographed with intensifying screens at −80°C for 7 d.

**Western blotting.** Cells were lysed in lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.5% NP40, 0.5% sodium deoxycholate, and Protease Inhibitor Cocktail Set III (EMD Biosciences, Inc.)]. We used an enhanced chemiluminescence (Amersham) Western blotting analysis system (GE Healthcare Bio-sciences), as previously described (15). A commercially available rabbit polyclonal antibody to human WDHD1 was purchased from ATLAS Antibodies AB and was proved to be specific to human WDHD1 by Western blot analysis using lysates of lung and esophageal cancer cell lines.

**Immunocytochemical analysis.** Immunocytochemical analyses were done as previously described (30), using 0.5 μg/mL of a rabbit polyclonal anti-WDHD1 antibody (ATLAS Antibodies AB) for detecting endogenous WDHD1 as a primary antibody and an Alexa 488–conjugated donkey anti-rabbit secondary antibody (Molecular Probe). Each stained specimen was mounted with Vectashield (Vector Laboratories, Inc.) containing 4′,6-diamidino-2-phenylindole and visualized with Spectral Confocal Scanning Systems (TSC SP2 AOB; Leica Microsystems).

**Immunohistochemistry and tissue microarray.** To investigate the WDHD1 protein in clinical samples that had been embedded in paraffin blocks, we stained the sections using ENVISION+ kit/horseradish peroxidase (DakoCytomation) in the following manner. Briefly, slides were immersed in Target Retrieval Solution and boiled at 108°C for 15 min in an autoclave for antigen retrieval. Rabbit polyclonal anti-WDHD1 antibodies (1.6 μg/mL; ATLAS Antibodies AB) were added to each slide after blocking of endogenous peroxidase and proteins, and the sections were incubated with horseradish peroxidase–labeled anti-rabbit IgG as the secondary antibody. Substrate-chromogen was added, and the specimens were counterstained with hematoxylin.

Tumor tissue microarrays were constructed with formalin-fixed 267 primary lung cancers and 283 primary esophageal cancers as described elsewhere (44–46). The tissue area for sampling was selected based on visual alignment with the corresponding H&E-stained section on a slide. Three, four, or five tissue cores (diameter, 0.6 mm; depth, 3-4 mm) taken from a donor tumor block were placed into a recipient paraffin block with a tissue microarrayer (Beecher Instruments). A core of normal tissue was punched from each case, and 5-μm sections of the resulting microarray block were used for immunohistochemical analysis. Three independent investigators semiquantitatively assessed WDHD1 positivity without prior knowledge of clinicopathologic data. Because the intensity of staining within each tumor tissue core was mostly homogenous, the positivity of WDHD1 staining was recorded by following criteria: negative (no appreciable staining in tumor cells) and positive (brown staining appreciable in the nucleus and cytoplasm of tumor cells). Cases were accepted as positive only if all reviewers independently defined them as such.

**Statistical analysis.** Statistical analyses were done using the StatView statistical program (SAS). Survival curves were calculated from the date of surgery to the time of death related to NSCLC or ESCC, or to the last follow-up observation. Kaplan-Meier curves were calculated for each relevant variable and for WDHD1 expression; differences in survival times among patient subgroups were analyzed using the log-rank test. Univariate and multivariate analyses were done with the Cox proportional hazard regression model to determine associations between clinicopathologic variables and cancer-related mortality. First, we analyzed associations between death and possible prognostic factors including age, gender, pT classification, and pN classification, taking into consideration one factor at a time. Second, multivariate Cox analysis was applied on backward (stepwise) procedures that always forced strong WDHD1 expression into the model, along with any and all variables that satisfied an entry level of a P value of <0.05. As the model continued to add factors, independent factors did not exceed an exit level of P < 0.05. Two-tailed P value of <0.05 were considered statistically significant.

**Cell growth assay.** COS-7 cells were plated at densities of 1 × 105 cells/100-mm dish, transfected with plasmids designed to express WDHD1 or mock plasmids. Cells were selected in medium containing 0.4 mg/mL of geneticin (Invitrogen) for 7 d, and cell viability was assessed by MTT assay (cell counting kit-8 solution; Dojindo Laboratories). The number of colonies stained with Giemsa was also counted by colony formation assay using colony counting software (ImageJ software 1.42, NIH).6

**RNA interference assay.** Small interfering RNA (siRNA) duplexes (Dharmacon, Inc.; 100 nmol/L) were transfected into a NSCLC cell line A549 and an esophageal cancer cell line TE9, using 30 μL of Lipofectamine 2000 (Invitrogen) as described (33). The transfected cells were cultured for 7 d and cell growth was evaluated by MTT and colony formation assays. siRNA duplexes against human WDHD1 and AKT1 used were as follows: si-WDHD1#1, siGenome duplexes 1 [D-019780-01], 5′-GAUCAGACACUGUG-CUAUUUA-3′; si-WDHD1#2: siGenome duplexes 2
Flow cytometry. Cells were collected in PBS and fixed in 70% cold ethanol for 30 min. After treatment with 100 μg/mL RNase (Sigma/Aldrich), the cells were stained with 50 μg/mL propidium iodide (Sigma/Aldrich) in PBS. Flow cytometry was done on a Becton Dickinson FACScan and analyzed by ModFit software (Verity Software House, Inc.). The cells selected from at least 20,000 ungated cells were analyzed for DNA content.

Live cell imaging. Cells were grown on a 35-mm glass-bottom dish in phenol red–free DMEM containing 10% FCS. Cells were transfected with siRNA and subjected to time lapse imaging using a computer-assisted fluorescence microscope (Olympus, LCV100) equipped with an objective lens (Olympus, UAPO 40×/0.90), with 50 μg/mL propidium iodide (Sigma/Aldrich) in PBS. Flow cytometry was done on a Becton Dickinson FACScan and analyzed by ModFit software (Verity Software House, Inc.). The cells selected from at least 20,000 ungated cells were analyzed for DNA content.
Fig. 2. Expression of WDHD1 in normal tissues and association of WDHD1 overexpression with poor prognosis for NSCLC and ESCC patients. A, Northern blot analysis of the WDHD1 transcript in 23 normal adult human tissues. A strong signal was observed in testis. B, immunohistochemical analysis of WDHD1 protein expressions in five normal tissues (liver, heart, kidney, lung, and testis) with those in lung SCC. WDHD1 was expressed abundantly in testis (mainly in nucleus and/or cytoplasm) and lung cancers, but its expression was hardly detectable in the remaining four normal tissues. C and D, association of WDHD1 expression with poor prognosis. Kaplan-Meier analysis of survival of patients with NSCLC (C, $P = 0.0403$ by the log-rank test) and ESCC (D, $P = 0.0426$ by the Log-rank test) according to expression of WDHD1.
a halogen lamp, a red LED (620 nm), a CCD camera (Olympus, DP30), differential interference contrast optical components, and interference filters. For differential interference contrast imaging, the red LED was used with a filter cube containing an analyzer. Image acquisition and analysis were done by using MetaMorph 6.13 software (Universal Imaging).

**In vitro kinase assay.** Flag-tagged WDHD1 protein that was exogenously expressed in COS-7 cells was immuno-precipitated using Flag-M2 agarose. The immunoprecipitant was confirmed to be WDHD1 by Western blotting using a mouse monoclonal anti–Flag-M2 antibody. The Flag-tagged WDHD1 protein was incubated with recombinant human AKT1 protein (rhAKT1; Invitrogen) in kinase buffer [20 mmol/L Tris (pH 7.5), 10 mmol/L MgCl2, 2 mmol/L MnCl2, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L DTT] supplemented with a mixture of protease inhibitors, 10 mmol/L NaF, 5 nmol/L microcystin.

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**Table 1. WDHD1 positivity and NSCLC patients' characteristics**

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>WDHD1 positive</th>
<th>WDHD1 negative</th>
<th>P: positive vs negative</th>
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<tbody>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>89 (33.3%)</td>
<td>27 (10.1%)</td>
<td>62 (23.2%)</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Male</td>
<td>178 (66.7%)</td>
<td>109 (40.8%)</td>
<td>69 (25.8%)</td>
<td></td>
</tr>
<tr>
<td><strong>Age (y)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;65</td>
<td>132 (49.4%)</td>
<td>58 (21.7%)</td>
<td>74 (27.7%)</td>
<td>0.0277*</td>
</tr>
<tr>
<td>≥65</td>
<td>135 (50.6%)</td>
<td>78 (29.2%)</td>
<td>57 (21.3%)</td>
<td></td>
</tr>
<tr>
<td><strong>Smoking</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonsmoker</td>
<td>88 (33.0%)</td>
<td>31 (11.6%)</td>
<td>57 (21.3%)</td>
<td>0.0004*</td>
</tr>
<tr>
<td>Smoker</td>
<td>179 (67.0%)</td>
<td>105 (39.3%)</td>
<td>74 (27.7%)</td>
<td></td>
</tr>
<tr>
<td><strong>Histologic type</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADC</td>
<td>157 (58.8%)</td>
<td>59 (22.1%)</td>
<td>98 (36.7%)</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Non-ADC</td>
<td>110 (41.2%)</td>
<td>77 (28.8%)</td>
<td>33 (12.4%)</td>
<td></td>
</tr>
<tr>
<td><strong>pT factor</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>114 (42.7%)</td>
<td>43 (16.1%)</td>
<td>71 (26.6%)</td>
<td>0.0002*</td>
</tr>
<tr>
<td>T2+T3</td>
<td>153 (57.3%)</td>
<td>93 (34.8%)</td>
<td>60 (22.5%)</td>
<td></td>
</tr>
<tr>
<td><strong>pN factor</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>208 (77.9%)</td>
<td>98 (36.7%)</td>
<td>110 (41.2%)</td>
<td>0.0264*</td>
</tr>
<tr>
<td>N1+N2</td>
<td>59 (22.1%)</td>
<td>38 (14.2%)</td>
<td>21 (7.9%)</td>
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</tbody>
</table>

**B. Cox's proportional hazards model analysis of prognostic factors in patients with NSCLCs**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Hazard ratio (95% CI)</th>
<th>Unfavorable/favorable</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Univariate analysis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WDHD1</td>
<td>1.668 (1.017-2.734)</td>
<td>Positive/negative</td>
<td>0.0425†</td>
</tr>
<tr>
<td>Age (y)</td>
<td>2.171 (1.313-3.590)</td>
<td>≥65/65&gt;</td>
<td>0.0025†</td>
</tr>
<tr>
<td>Gender</td>
<td>2.135 (1.198-3.803)</td>
<td>Male/Female0.0</td>
<td>100†</td>
</tr>
<tr>
<td>Smoking</td>
<td>1.870 (1.075-3.253)</td>
<td>Smoker/non-Smoker</td>
<td>0.0267†</td>
</tr>
<tr>
<td>Histologic type</td>
<td>2.493 (1.516-4.101)</td>
<td>Non-ADC/ADC</td>
<td>0.0003†</td>
</tr>
<tr>
<td>pT factor</td>
<td>3.722 (2.026-6.838)</td>
<td>T2+T3/T1</td>
<td>&lt;0.0001†</td>
</tr>
<tr>
<td>pN factor</td>
<td>4.430 (2.718-7.220)</td>
<td>N1+N2/N0</td>
<td>&lt;0.0001†</td>
</tr>
<tr>
<td><strong>Multivariate analysis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WDHD1</td>
<td>0.816 (0.467-1.424)</td>
<td>Positive/negative</td>
<td>0.4741</td>
</tr>
<tr>
<td>Age (y)</td>
<td>1.806 (1.084-3.011)</td>
<td>≥65/65&gt;</td>
<td>0.0233†</td>
</tr>
<tr>
<td>Gender</td>
<td>1.263 (0.568-2.811)</td>
<td>Male/female</td>
<td>0.5669</td>
</tr>
<tr>
<td>Smoking</td>
<td>1.290 (0.619-2.685)</td>
<td>Smoker/non-smoker</td>
<td>0.4966</td>
</tr>
<tr>
<td>Histologic type</td>
<td>1.628 (0.891-2.973no)</td>
<td>Non-ADC/ADC</td>
<td>0.1127</td>
</tr>
<tr>
<td>pT factor</td>
<td>2.291 (1.199-4.380)</td>
<td>T2+T3/T1</td>
<td>0.0121†</td>
</tr>
<tr>
<td>pN factor</td>
<td>3.723 (2.254-6.148)</td>
<td>N1+N2/N0</td>
<td>&lt;0.0001†</td>
</tr>
</tbody>
</table>

(Continued on the following page)
Results

**WDHD1 expression in lung and esophageal cancers and normal tissues.** To identify novel molecules such as cancer testis antigens that were highly transactivated in a large proportion of lung and esophageal cancers, but scarcely expressed in normal tissues, we had applied cDNA microarray analysis, and identified elevated expression (3-fold or higher) of the WDHD1 transcript in the majority of lung cancer and ESCC tissue samples examined. Moreover, WDHD1 showed testis-specific expression in normal tissues. We confirmed its overexpression by means of semi-quantitative RT-PCR experiments in 14 of 15 lung cancer tissues, in all of 15 lung cancer cell lines, in 6 of 10 ESCC tissues, and in 8 of 11 ESCC cell lines (Fig. 1A and B). We also confirmed its elevated expression in another set of primary tumor tissues (six of six NSCLCs and five of six ESCCs); however, its expression was hardly detectable in the remaining four normal tissues (Fig. 2B).

**Association of WDHD1 expression with poor prognosis for patients with NSCLC or ESCC.** To investigate the biological and clinicopathologic significance of WDHD1 in pulmonary and esophageal carcinogenesis, we carried out immunohistochemical staining on tissue microarray containing tissue sections from 267 NSCLC and 283 ESCC cases that underwent surgical resection. WDHD1 staining with polyclonal antibody specific to WDHD1 was mainly observed in the nucleus as well as the cytoplasm of tumor cells, but its expression was hardly detectable in the remaining four normal tissues (Fig. 2B).

## Table 1. WDHD1 positivity and NSCLC patients’ characteristics (Cont’d)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Odds ratio</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (male/female)</td>
<td>2.130</td>
<td>0.0339( ^{\text{s}} )</td>
</tr>
<tr>
<td>Age (≥65 y/&lt;65 y)</td>
<td>1.335</td>
<td>0.2914</td>
</tr>
<tr>
<td>Smoking (smoker/nonsmoker)</td>
<td>1.285</td>
<td>0.4739</td>
</tr>
<tr>
<td>Histologic type (non-ADC/ADC)</td>
<td>2.437</td>
<td>0.0033( ^{\text{s}} )</td>
</tr>
<tr>
<td>pT factor ((T_2-3/T_1))</td>
<td>1.397</td>
<td>0.2690</td>
</tr>
<tr>
<td>pN factor ((N_1-2/N_0))</td>
<td>1.971</td>
<td>0.0487( ^{\text{s}} )</td>
</tr>
</tbody>
</table>

Abbreviations: Non-ADC, squamous cell carcinoma plus large-cell carcinoma and adenosquamous cell carcinoma; 95% CI, 95% confidence interval.

\( ^{\text{s}} P < 0.05 \) (Fisher’s exact test).

\( ^{\text{t}} P < 0.05 \).

\( ^{\text{t}} \) Selected factors from Table 1A that were significantly associated with WDHD1 positivity.

\( ^{\text{s}} P < 0.05 \).
size, T1 versus T2+T3, pN stage (node status, N0 versus N1 +N2), histologic type (non-ADC versus ADC), and WDHD1 expression (positive versus negative). All those parameters were significantly associated with poor prognosis (Table 1B). In multivariate analysis, WDHD1 status did not reach the statistically significant level as an independent prognostic factor for surgically treated lung cancer patients enrolled in this study ($P=0.4741$), whereas pT and pN stages as well as age did so (Table 1B). The result might be due to the relevance of WDHD1 expression mainly to the other prognostic factors—male gender, non-ADC histology, and/or presence of lymph node metastasis in lung cancer—probably because multivariate logistic regression analysis for the six clinicopathologic parameters that were significantly associated with WDHD1 positivity in lung cancer (Table 1A) also determined that these three factors were independent features associated with WDHD1 expression ($P=0.0339, 0.0033$, and $0.0487$, respectively; Table 1C).

Of the 283 ESCC cases examined, WDHD1 was stained positively in the nucleus as well as the cytoplasm of tumor cells in 175 cases (61.8%) and negatively in 108 cases (38.2%), whereas their adjacent normal esophagus tissues were not stained (Supplementary Fig. S1D and E; Table 2A). ESCC patients whose tumors showed WDHD1 expression revealed shorter tumor-specific survival periods compared with those with absent WDHD1 expression ($P=0.0426$ by log-rank test; Supplementary Table S3B; Fig. 2D). We also applied univariate analysis to evaluate associations between ESCC patient prognosis and several factors including age, gender, pT stage (tumor depth, T1 versus T2+T3), pN stage (node status, N0 versus N1+N2), and WDHD1 status (positive versus negative). All those parameters except for age were significantly associated with poor prognosis (Table 2B). Multivariate analysis using a Cox proportional hazard factors determined that WDHD1 ($P=0.0104$) as well as other three factors (male gender, larger tumor size, and lymph node metastasis) were independent prognostic factors for surgically treated ESCC patients (Table 2B).

**Effects of WDHD1 on cell growth and cell cycle progression.** To assess whether WDHD1 plays an essential role...
in cell growth or survival, we carried out a colony formation assay of COS-7 transfected with WDHD1 expression plasmids and confirmed the growth-promoting activity of cells overexpressing WDHD1, compared with those transfected with mock vectors (Supplementary Fig. S2). We further constructed several siRNA expression oligonucleotides specific to WDHD1 sequences and transfected them into lung cancer A549 cells as well as esophageal cancer TE9 cells that endogenously expressed WDHD1 at high levels. Knockdown effects were confirmed by semi-quantitative RT-PCR when we used si-WDHD1-#1 and si-WDHD1-#2 (Fig. 3A). MTT assays and colony formation assays revealed a drastic reduction in the number of cells transfected with si-WDHD1-#1 and #2 (Supplementary Fig. S3; Fig. 3B). Flow cytometric analysis at 24 to 72 hours after the transfection of si-WDHD1 to the A549 and TE9 cells revealed the decrease of the number of cells at S phase and the increase of that in G0-G1 phase (Supplementary Fig. S4A; Fig. 3C). To further investigate the effect of WDHD1 on the cell cycle progression, we synchronized A549 and TE9 cells (5 × 10^5 cells/100-mm dish) transfected with siRNA for si-WDHD1. The flow cytometric and Western blot analyses at 0, 4.5, and 9 hours after removal of aphidicolins showed that the number of the A549 and TE9 cells in G0-G1 phase was increased and the progression to S phase was delayed (Supplementary Fig. S4B and C). To further clarify the effect of WDHD1 knockdown on cellular morphology and cell cycle, we examined A549 cells transfected with siRNA for WDHD1 using time lapse microscopy. Although the cell division was observed at about every 10 hours in control cells, the WDHD1 knocked down cells started cell division slowly and died shortly after cell division (Fig. 3D).

**Phosphorylation of WDHD1.** WDHD1 protein was detected as double bands by Western blotting, indicating a possible modification of the WDHD1 protein. Therefore, we first incubated extracts from A549 cells that overexpressed endogenous WDHD1 and also COS-7 cells transfected with WDHD1-expressing plasmids in the presence or absence of protein phosphatase (New England Biolabs), and analyzed the molecular size of WDHD1 protein by Western blot analysis. The measured weight of the majority of both endogenous and exogenous WDHD1 protein in the extracts treated with phosphatase was smaller than that in the untreated cells. The data indicated that WDHD1 was possibly phosphorylated in cells (Supplementary Fig. S5A). Immunoprecipitation of WDHD1 with anti-WDHD1 antibody followed by immunoblotting with pan-phospho–specific antibodies indicated phosphorylation of endogenous WDHD1 at its serine and tyrosine residues (Supplementary Fig. S5B).

**Involvement of WDHD1 in PI3K/AKT pathway.** To elucidate the importance of WDHD1 phosphorylation in cancer cells, we next screened the candidate kinases for WDHD1 by referring possible phosphorylation sites on WDHD1 and found a consensus phosphorylation site for AKT kinase (R-X-R-X-X-S374; ref. 47). PI3K/AKT pathway is well known to be activated in a wide range of tumor types, and this triggers a cascade of responses, from cell growth and proliferation to survival, motility, epithelial-mesenchymal transition, and angiogenesis (48). We therefore examined whether WDHD1 could be involved in the PI3K and/or AKT pathway. We first immunoblotted, using PAS antibody, the Flag-tagged WDHD1 protein that was exogenously expressed in COS-7 cells and immunoprecipitated using Flag-M2 agarose, and detected the positive band that represented possibly phosphorylation by endogenous AKT (Fig. 4A). In vitro kinase assay using the Flag-tagged WDHD1 immunoprecipitant as a substrate and recombinant human AKT1 protein (rhAKT1) as a kinase with subsequent immunoblotting with PAS antibody also proved the direct phosphorylation of WDHD1 by AKT1 (Fig. 4B). To examine the effect of AKT1 on WDHD1 protein function in cancer cells, we measured the level of endogenous WDHD1 protein after transfection of siRNA for AKT1 to LC319 cells or after treatment of the cells with various concentrations of LY294002 (0-20 μmol/L for 24 hours), a specific inhibitor of the catalytic subunit of PI3K. Total amount of WDHD1 as well as phosphorylated WDHD1 was significantly decreased by LY294002 treatment or introduction of siRNA for AKT1, indicating a possibility that WDHD1 protein stability is regulated by the PI3K/AKT signaling (Fig. 4C and D).

**Discussion**

We performed a genome-wide expression profile analysis of 101 lung cancers and 19 ESCC cells after enrichment of cancer cells by laser microdissection, using a cDNA microarray containing 27,648 genes or expressed sequence tags (7–12). Through the analyses, we identified a number of genes that could be potentially good candidates for the development of novel diagnostic markers, therapeutic drugs, and/or immunotherapy (13–37). In this study, we selected WDHD1 as a good candidate for cancer biomarker(s) for lung cancer and/or ESCC, and provided evidences for its possible role in human carcinogenesis.

WDHD1, a homologue of Ctf4/Mcl1 in Saccharomyces cerevisiae and Schizosaccharomyces pombe, encodes a putative 1129 amino acid protein with high-mobility group box domains and a WD-repeat domain. Ctf4/Mcl1 has been described as a part of a replisome progression complex that associates with the GINS complex as well as with DNA polymerase/primase (49). To maintain the genome integrity, chromosomal DNA is precisely replicated only once per cell cycle. Initiation of chromosomal replication starts by the binding of several factors to replication origins, and the interactions among these factors are crucial for subsequent processes (50). Ctf4/Mcl1 physically interacts with DNA pol α and induces the association of Pol α to chromatin, and plays a critical role in lagging strand synthesis and Okazaki fragment processing (40). Recent studies showed that WDHD1 interacts with MCM10 and is essential for DNA synthesis, suggesting its role as a replication initiation factor (41). The function of Ctf4/Mcl1 in chromosome replication, cohesion, and segregation...
Fig. 3. Growth-promoting effect of WDHD1. A and B, inhibition of growth of a lung cancer cell line A549 (left) and an esophageal cancer cell line TE9 (right) by siRNAs against WDHD1. A, WDHD1 gene knockdown effect in A549 and TE9 cells by two si-WDHD1 (si-WDHD1-#1 and si-WDHD1-#2) and two control siRNAs (si-EGFP and si-LUC), analyzed by semiquantitative RT-PCR. B, MTT assays of A549 and TE9 cells transfected with si-WDHD1s or control siRNAs. Columns, relative absorbance of triplicate assays; bars, SD. C, flow cytometric analysis of lung cancer cells transfected with si-WDHD1. A549 cells were transfected with si-WDHD1-#1 or si-LUC, and collected at 24, 48, and 72 h after transfection for flow cytometry. The numbers besides the panels indicate the percentage of cells at each phase.
was reported to date, but the overexpression of WDHD1 in human cancer cells and its possible role in human carcinogenesis have not been described.

In this study, we obtained important clinicopathologic and biological evidences supporting the significance of WDHD1 transactivation in human carcinogenesis. We showed through our tissue microarray experiments that NSCLC and ESCC patients with WDHD1-positive tumors had shorter cancer-specific survival periods than those with WDHD1-negative tumors. In addition, the enhanced expression of WDHD1 significantly promoted growth of mammalian cells. Moreover, WDHD1 knockdown by siRNA in cancer cells delayed S-phase entry and progression, and resulted in cell death right after cell division. The data imply the importance of WDHD1 in cancer cell cycle progression, although further detailed analyses of WDHD1 function in coordinated maintenance of the cancer genome integrity are required.

Our study also described the involvement of WDHD1 activation in cancer cell signaling. WDHD1 seemed to be phosphorylated and stabilized by AKT1. PI3K/AKT signaling is important for cell proliferation and survival (48). In addition, AKT1 phosphorylation frequently occurs in various human cancers, and has been recognized as a risk factor for early disease recurrence and poor prognosis (48). Our data indicated that inhibition of PI3K/AKT pathway using LY294002 or siRNA for AKT1 decreased the level of total and phosphorylated WDHD1 in lung cancer cells. Because these results indicate that WDHD1 plays a significant role in cancer cell growth/survival as one of the components of the PI3K/AKT pathway, selective targeting of functional interaction between AKT1 and WDHD1 could be a promising therapeutic strategy. Further analyses of the mechanism of growth suppression by specific inhibition of WDHD1 phosphorylation by AKT1 may be of the great benefit toward the development of new types of anticancer agents.

From the result of Northern blot and immunohistochemical analyses, WDHD1 was expressed only in testis and cancer cells. Cancer testis antigens have been recognized as a group of highly attractive targets for cancer vaccine treatment (34–37). Although other factors, such as the in vivo spontaneous immunogenicity of the protein, are also important and further examination will be necessary, WDHD1 oncoantigen may be useful for screening of HLA-restricted epitope peptides for cancer vaccine that
Fig. 4. Possible regulation of WDHD1 stability by its phosphorylation through PI3K/AKT signaling. A, immunoprecipitant of Flag-tagged WDHD1 in COS-7 cells transfected with Flag-tagged WDHD1-expressing plasmid was detected with anti-PAS antibody or anti–Flag-M2 antibody. B, in vitro phosphorylation of Flag-tagged WDHD1 protein by recombinant human AKT1 (rhAKT1). C, reduction of WDHD1 protein by PI3K inhibition with LY294002. LC319 were treated with PI3K inhibitor LY294002 in concentrations of 0 or 20 μmol/L for 24 h and served for Western blot analysis. D, reduction of WDHD1 protein by AKT1 inhibition with siRNA against AKT1. LC319 were transfected with siRNA for AKT1 or EGFP and served for Western blot analysis.
can induce specific immune responses by cytotoxic T cells against WDHD1-positive cancer cells. Because WDHD1 expression could have pivotal functions in cancer cell survival, vaccination with the peptides from this protein should reduce the risk of the emergence of immune escape variant tumors that have lost their antigen expression.

In conclusion, WDHD1 was overexpressed in the great majority of lung and esophageal cancer tissues, and it is likely to play significant roles in cancer cell growth and/or survival. The data indicate WDHD1 to be a potential therapeutic target and a candidate biomarker for patients with lung and esophageal cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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